

1 **The toad fly *Lucilia bufonivora*: its evolutionary status and molecular identification**

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13

14 **Abstract.** The blowfly genus *Lucilia* is composed largely of saprophages and facultative myiasis
15 agents, including the economically important species *Lucilia cuprina* and *Lucilia sericata*. Only one
16 species is generally recognised as an obligate agent of myiasis, *Lucilia bufonivora* Moniez, and this
17 is an obligate parasite of toads. *Lucilia silvarum* (Meigen), a sister species, behaves mainly as a
18 carrion breeder, however, it has also been reported as a facultative parasite of amphibians.
19 Morphologically, these species are almost identical and historically this has led to misidentification,
20 taxonomic ambiguity and a paucity of studies of *L. bufonivora*. In this study, dipterous larvae were
21 analysed from toad myiasis cases from the UK, The Netherlands and Switzerland, together with
22 adult specimens of fly species implicated in amphibian parasitism: *L. bufonivora*, *L. silvarum* and
23 *Lucilia elongata* (from North America). Partial sequences of two genes, *cox1* and *ef1a*, were
24 amplified. Seven additional blowfly species were analysed as outgroups. Bayesian inference trees
25 of *cox1*, *ef1a* and a combined-gene dataset were constructed. All larvae isolated from toads were
26 identified as *L. bufonivora* and no specimens of *L. silvarum* were implicated in amphibian myiasis.
27 This study confirms *L. silvarum* and *L. bufonivora* as distinct sister species and provides
28 unambiguous molecular identification of *L. bufonivora*.

29

30 **Key words.** Myiasis, obligate parasitism, Calliphoridae, *Bufo bufo*, cytochrome *c* oxidase subunit 1,
31 Elongation factor 1 alpha

32

33 Introduction

34 The cosmopolitan genus of calliphorid blowflies, *Lucilia*, is composed largely of saprophages and
35 facultative agents of myiasis, the latter showing species-specific differences in their propensity to
36 infest living hosts. Of most economic importance within the genus are *Lucilia cuprina*
37 (Wiedemann) and *Lucilia sericata* (Meigen), which are primary agents of sheep myiasis in many
38 areas of the world. Only one species is believed to be an obligate agent of myiasis, *Lucilia*
39 *bufonivora* Moniez, which has a high host-specificity for anurans. Eggs are laid on the living host
40 and, after hatching, the first stage larvae migrate to the nasal cavities where larval development
41 takes place (Fig. 1), usually resulting in the death of the amphibian host (Zumpt, 1965).
42 *L. bufonivora* has been reported as the cause of myiasis in a range of amphibian hosts, however,
43 most reports relate to infestations of the common toad, *Bufo bufo* (Weddeling & Kordges, 2008;
44 Diepenbeek & Huijbregts, 2011; Martín *et al.*, 2012). This blowfly is widely distributed in Europe
45 (Rognes, 1991; Verves & Khrokalo, 2010) and Asia (Fan *et al.*, 1997), and recently adult specimens
46 of *L. bufonivora* have been reported in North America and Canada (Tantawi & Whitworth, 2014).

47 *Lucilia silvarum* (Meigen) is another widely distributed blowfly species in the Palearctic
48 (Schumann, 1986) and the Nearctic (Hall, 1965). It lives mainly as a carrion breeder in the
49 Palearctic (Zumpt, 1956), however, there are several reports of *L. silvarum* being involved in
50 amphibian myiasis in North America (Hall, 1948; Bolek & Coggins, 2002; Bolek & Janovy, 2004;
51 Eaton *et al.*, 2008) and therefore it is usually considered a facultative rather than an obligate parasite
52 (Nuorteva, 1963); there is no reliable evidence of the involvement of this species in amphibian
53 myiasis in Europe.

54 While most cases of toad myiasis by *L. bufonivora* have been reported to occur in the nasal
55 cavities of their host (Diepenbeek & Huijbregts, 2011; Martín *et al.*, 2012), toad myiases due to
56 *L. silvarum* have been reported to occur in the back, neck, legs and parotid glands of the host; there
57 are no reports of *L. silvarum* developing in the nasal cavities (Bolek & Coggins, 2002; Bolek &
58 Janovy, 2004). Despite this apparent behavioural difference, the adults of these two closely related

59 blowfly species are almost identical on the basis of morphology and reliable identification requires
60 examination of the male genitalia or the female ovipositor. Morphological identification and
61 differentiation of the larval stages is even more problematic, and Zumpt (1965) argued that in
62 Europe most records of toad myiasis thought to have been caused by *L. silvarum* should probably
63 be attributed to *L. bufonivora*.

64 Due to their morphological similarity, the taxonomic status of *L. bufonivora* and *L. silvarum*
65 has been debated over many decades; indeed, Townsend (1919) proposed a new genus, *BufoLucilia*,
66 which included *L. bufonivora* as the type species, along with *L. silvarum*. Subsequently, Hall (1948)
67 included *Lucilia elongata* Shannon in this genus, which has also been reported as a facultative
68 amphibian parasite in North America (James & Maslin, 1947; Bolek & Janovy, 2004). More
69 recently, the genus *BufoLucilia* was dismissed as a synonym of *Lucilia* by Rognes (1991), although
70 it continues to be recognised as a genus or subgenus by a number authors (e.g. Kraus, 2007; Verves
71 & Khrokalo, 2010; Draber-Mońko, 2013). However, while several studies provide strong support
72 for the grouping of *L. bufonivora* and *L. silvarum* as closely related sister species (e.g. Stevens &
73 Wall, 1996a; McDonagh & Stevens, 2011), recognition of genus *BufoLucilia* would leave other
74 *Lucilia* species in a heterogeneous and paraphyletic group, as observed with some other proposed
75 (but poorly supported) genera, for example, *Phaenicia* (Stevens & Wall, 1996a).

76 Here, we utilise sequence data from the mitochondrial protein-coding gene cytochrome c
77 oxidase subunit I (*cox1*) and the nuclear gene elongation factor 1 alpha (*ef1a*) to facilitate
78 unambiguous identification of *L. bufonivora* larvae infesting live toads and we identify the causal
79 agent of obligate amphibian myiasis. Additionally, we confirm the hypothesis that *L. bufonivora*
80 and *L. silvarum* are distinct sister species, and we discuss the evolutionary relationships between the
81 closely related taxa associated with amphibian myiasis.

82

83 **Materials and methods**

84 *Adult and larval specimens*

Larval specimens putatively identified as *L. bufonivora* were sampled from 16 separate toad myiasis cases from six different locations in Britain (8 cases), four locations in The Netherlands (7 cases) and one site in Switzerland (1 case) (Table 1, Fig. S1). Four adult specimens of *L. bufonivora* were also analysed, two from southern Germany and two collected with the aid of baited traps in The Netherlands (Table 2, Fig. S1). Five adult specimens of *L. silvarum* were analysed, including three from the UK, one from the USA and one from The Netherlands. A specimen of *L. elongata* from Alberta, Canada was also added to facilitate further exploration of the evolutionary relationships across the broader group of fly species reported as amphibian parasites.

For comparative purposes, adult specimens of seven other *Lucilia* species were also analysed (Table 2, Fig. S1). Specimens were collected in the UK and The Netherlands using liver-baited traps and identified using keys by van Emden (1954). Additionally, two new specimens of adult *Lucilia mexicana* from Chapingo, Mexico were analysed (Table 2). Sequence data for specimens of *L. silvarum*, *L. sericata*, *L. cuprina* and *L. illustris* and *Lucilia ampullacea* were obtained from EMBL/GenBank and also included in the analysis. Three adult samples of *Calliphora vicina* collected in the UK and Switzerland were included as outgroup taxa. All specimens were stored in 100% ethanol at 4°C prior to analysis.

DNA extractions and PCR procedures

Thoracic muscle of adult specimens was used for DNA extraction to avoid contamination with ingested protein, eggs or parasites. To avoid potential contamination from larval gut contents, the anterior and posterior ends of larvae were used for DNA extraction from LII and LIII life stages, while whole specimens were used if samples were LI; live larvae were maintained on damp filter paper for 3–6 hours prior to storage in ethanol to allow them to evacuate their gut contents. DNA extractions were carried out using a QIAGEN DNeasy® Blood and Tissue Kit (Qiagen GmbH, Germany) according to manufacturer's instructions.

110 DNA was extracted as total nucleic acid and subjected to PCR to amplify the cytochrome
111 oxidase I (*coxI*) region of the mitochondrial protein-coding gene and the EF1-EF4 region of the
112 nuclear protein-coding gene elongation factor 1 alpha (*eflα*). Universal insect primers previously
113 published (Table 3) were used. The PCR protocol published by Folmer *et al.* (1994) was modified
114 to amplify *coxI* and *eflα* (EF1-EF4 region) with the following cycling conditions: 94°C for 5 min,
115 followed by 35 cycles of 95°C for 30 s, 50°C (*coxI*) or 48°C (EF1-EF4) for 30 s, 72°C for 1 min,
116 and a final step of 72°C for 1 min. A negative control (no template DNA) was included in each set
117 of PCR amplifications. PCR products were separated by gel electrophoresis and bands were
118 visualized by ethidium bromide staining. Targeted bands of *coxI* were cut out and purified using a
119 QIAquick® Gel Extraction Kit (Qiagen GmbH, Germany). Successful EF1-EF4 products were
120 purified using 0.5 μL of Exonuclease I and 0.5 μL of Antarctic phosphatase per 20 μL of PCR
121 product. A total of 658 bp of the *coxI* region were amplified in a single fragment with primers
122 HCO2198 and LCO1490. A fragment of 638 bp of the *eflα* region was amplified with primers EF1
123 and EF4. Purified PCR products were sequenced using commercial sequencing facilities,
124 EUROFINS® (*eflα*) and GENEWIZ® (*coxI*).

125

126 *Sequence alignment*

127 The quality of the sequences was checked and edited manually for both forward and reverse
128 fragments; sequences were then assembled into a single consensus sequence using BioEdit
129 software. Each consensus sequence was checked against previously published sequences in
130 EMBL/GenBank using BLAST. Multiple sequence alignment was carried out using BioEdit
131 implementing the CLUSTALW algorithm.

132

133 *Phylogenetic analysis*

134 The best-fitting nucleotide substitution model for each dataset was selected using
135 jModelTest (Posada, 2008) (TreNef + I was selected for the EF1-EF4 dataset; TIM3 + I +G was

136 selected for *cox1*). Prior to Bayesian inference analyses the best-fitting model selected for each gene
137 was implemented by changing the default settings (*nst*, *rates*, *ngammacat*, *statefreqpr*, *revmat*,
138 *shapepr* and *pinvarpr*) in the software MrBayes v3.2.6 (Huelsenbeck & Ronquist, 2001)
139 phylogenetic analysis was then carried out implementing MCMC starting from two independent
140 analyses simultaneously, each with three heated chains and one cold chain, they were run for 10,000
141 generations sampling every 10 generations. Analyses were stopped when the critical value for the
142 topological convergence diagnostic fell below the default threshold (0.01). A fraction (0.25) of the
143 sampled values were discarded (*burninfrac* = 0.25) when the convergence diagnostics were
144 calculated. Substitution model parameters (*sump*) and branch lengths (*sumt*) were summarized; tree
145 topology was then calculated with the remaining data by constructing a majority-rule consensus
146 tree.

147 A combined-gene analysis was also carried out with a partitioned dataset; model parameters
148 for each gene were implemented separately (unlinked), allowing each gene to evolve under different
149 rates. An incongruence length difference test (ILD) was run in PAUP*4.0a152 to test phylogenetic
150 congruence and to quantify the differences in topology between the single-gene trees. Analysis was
151 conducted on a partitioned dataset with the combined dataset (*efla* and *cox1*).

152

153

154 Results

155 *Molecular identification of Lucilia bufonivora*

156 All 20 larval specimens from the 16 infestations studied (Table 1) gave nuclear and
157 mitochondrial sequence data consistent with BLAST searches for *Lucilia bufonivora*. Additionally,
158 molecular data reaffirmed the identity of adult fly samples identified as *L. bufonivora* on the basis
159 of morphology. All *L. bufonivora* samples were grouped together in a single unstructured clade in
160 all phylogenies (Fig. 2, Fig. 3).

161

162 *Single-gene phylogenies*

163 In both single-gene phylogenies all amphibian parasite taxa grouped together. In the *eflα*-
164 based phylogeny amphibian parasite taxa formed a monophyletic clade (Fig. 2a); in the *cox1*-based
165 phylogeny *L. bufonivora* and *L. elongata* formed a monophyletic clade, while *L. silvarum* was
166 paraphyletic and incorporated *L. richardsi* (Fig. 2b). Within the amphibian parasite group (in each
167 single gene phylogeny) all *L. bufonivora* specimens analysed were classified together in a well-
168 supported monophyletic clade (Fig. 2a, b) with minimal intra-specific variation (only one English
169 specimen, Lbufo17, showed minor variation). However, analysis of *eflα*-sequence data did not
170 show clear distinction of *L. elongata* (a North American species) from *L. silvarum* (Fig. 2a),
171 although within this grouping both USA samples of *L. silvarum* (Sacramento and San Francisco)
172 were placed together with strong support. In the *cox1* phylogeny (Fig. 2b) *L. silvarum* samples from
173 the USA also grouped together with strong support, but were placed apart from European
174 *L. silvarum*, suggesting relatively high intra-specific variation in *L. silvarum*.

175 The placement of other *Lucilia* spp. relative to the amphibian parasite taxa was essentially as
176 described previously (McDonagh & Stevens 2011). All sequences of *Calliphora vicina* analysed
177 grouped together in the same outgroup clade.

178

179 *Combined-gene phylogeny*

180 The ILD test detected incongruence between the two genes used in this study ($P = 0.01$);
181 nonetheless, Bayesian inference analysis of a combined partitioned dataset produced a phylogeny
182 with generally strong posterior probabilities (Fig. 3). All *L. bufonivora* samples were grouped in a
183 single clade as a sister species to *L. elongata*. As observed in the *cox1* tree, a monophyletic
184 European *L. silvarum* group (GBR + NDL) was recovered, with *L. richardsi* grouped as its sister
185 taxon (Fig. 3); again, both American specimens of *L. silvarum* were placed outside of this group as
186 sister taxa with high support values. Both sheep blowfly species, *L. sericata* and *L. cuprina*, were
187 recovered as a monophyletic group with strong support. The closely related species *L. illustris* and

188 *L. caesar* were recovered as sister species, however, this combined-gene analysis placed
189 *L. mexicana* more closely related to the *L. caesar* group than the *L. ampullacea* clade. Subfamily
190 relationships of Luciliinae were recovered with strong posterior probability (1), grouping all
191 *C. vicina* samples as an outgroup and differentiating subfamily Calliphorinae from Luciliinae with
192 strong support (Fig. 3).

193

194 Discussion

195 Using mitochondrial data (*cox1*) McDonagh & Stevens (2011) differentiated *L. bufonivora* from
196 *L. silvarum* and placed them as separate sister species. However, in the same study both species
197 were placed in the same clade using *ef1a* and *28S rRNA* as phylogenetic markers, the latter failing
198 to classify them as distinct species. In this study, the EF1-EF4 region of the protein-coding nuclear
199 gene *ef1a* showed just a single nucleotide difference between the sequence data of *L. silvarum* and
200 *L. bufonivora*; however, Bayesian inference analysis showed clear groupings, identifying them as
201 distinct sister species. Addition of data from the North American amphibian parasite *L. elongata*,
202 another putatively closely related taxon, allowed an even clearer understanding of the evolutionary
203 relationships between *L. silvarum* and *L. bufonivora*, resulting in the differentiation of them as
204 distinct sister species. The *ef1a* tree supported the suggestion that *L. bufonivora* has diverged
205 relatively recently from its sister taxon *L. silvarum* (Stevens & Wall, 1996a). The *cox1*-based
206 phylogeny showed clear relationships and distinction between *L. bufonivora* and *L. silvarum*, a
207 finding reiterated in the combined-gene tree. It is probable that in the combined-gene tree a stronger
208 signal in the mtDNA data (*cox1*) is driving the clear distinction and is dominating the weaker
209 phylogenetic signal of the nuclear data (*ef1a*). The low signal present in the *ef1a* sequence data
210 accords with the relatively slow rate of evolution reported previously in this nuclear gene
211 (McDonagh & Stevens, 2011) compared with that reported in the majority of insect mitochondrial
212 genes (McDonagh *et al.*, 2016). Indeed, *cox1* has been widely used in blowfly systematics (Otranto
213 & Stevens, 2002; Stevens *et al.*, 2002; Wells *et al.*, 2002) and due to generally higher rates of

sequence change in mtDNA it is expected to reach reciprocal monophyly before nuclear genes (Funk & Omland, 2003; Dowton, 2004; Lin & Danforth, 2004). As such, mitochondrial sequence data (e.g. *cox1*) are useful for inferring the relationships of recently diverged species (Stevens & Wall, 1997; Shao & Barker, 2006), and our results reaffirm this, suggesting that *L. bufonivora* is clearly a separate but closely related species to *L. silvarum*. Taken together, such findings call into question the utility of apparently slowly evolving genes such as *eflα* for evolutionary analysis of relatively recently diverged Diptera. As such, future studies of this group may be advised to consider alternative nuclear genetic markers evolving at a rate better suited to the question(s) being asked. For example, Williams & Villet (2013) showed the *period* gene and a nuclear rRNA locus to be well-suited to elucidating the extent of hybridisation between two closely related *Lucilia* species (*L. cuprina* and *L. sericata*); moreover, their use of two nuclear loci overcame some of the problems of species determination and accurate phylogenetic reconstruction associated with ancient mitochondrial introgression and potentially recent hybridisation events which have unquestionably disrupted mtDNA-based blowfly phylogenies (Stevens & Wall, 1996b; Stevens *et al.*, 2002). In short, blowfly phylogenetic analyses do need to employ nuclear markers, but it is apparent that *eflα* may not be the ideal locus for elucidating relationships between closely related blowfly taxa.

Molecular analysis of different populations of *L. bufonivora* from across Europe, detected no intra-specific differences in mitochondrial sequence data, while the nuclear gene *eflα* also exhibited only minimal intra-specific sequence variation (Fig. 2a). However, in *L. silvarum* marked intra-specific variation in both nuclear and mitochondrial sequence data was observed between European and North American populations of this fly; recent phylogenetic analysis of populations of this species from the USA and Germany also showed a high degree of intra-specific difference (Williams *et al.*, 2016). In the current study, intra-specific variation was also observed between European samples, with UK *L. silvarum* differing from a Dutch specimen of the same species. In contrast, a lack of significant variation in both nuclear and mitochondrial genes in the different European populations of *L. bufonivora* analysed suggests that it may be a recently diverged species

240 that has accumulated less molecular variation. Further studies would be of value, particularly to
241 explore the differences between European and North American populations of *L. bufonivora* (e.g.
242 Tantawi & Whitworth, 2014).

243 Even when both species have been reported as amphibian parasites (Baumgartner, 1988),
244 *L. bufonivora* has never been observed breeding in carrion. In contrast, its sister species *L. silvarum*
245 is reported mainly as a common carrion-breeding species in Europe (Rognes, 1991), with no
246 confirmed records of parasitism in amphibians due to it in this region (Diepenbeek & Huijbregts,
247 2011; Fremdt *et al.*, 2012). In North America, however, there have been several reports of
248 amphibian myiasis cases apparently involving *L. silvarum* (Bolek & Coggins 2002; Bolek & Janovy
249 2004; Eaton *et al.*, 2008). The phylogeny constructed from the combined dataset characterised
250 *L. silvarum* from the USA as more closely related to *L. bufonivora* than to *L. silvarum* from Europe.
251 This finding is congruent with the reported amphibian parasitic behaviour of North American
252 *L. silvarum*, and reiterates the significance of the relatively high intra-specific variation present
253 between European and North American populations of *L. silvarum*, which in turn reflects the fact
254 that very different larval feeding strategies can be exhibited even between closely related blowfly
255 taxa (Stevens, 2003; Stevens & Wallman, 2006).

256 Using the nuclear marker *efla*, amphibian parasitism in *Lucilia* appears as a monophyletic
257 trait with the inclusion of *L. bufonivora*, *L. silvarum* and *L. elongata*. However, in the combined-
258 gene and *cox1* trees this group becomes paraphyletic due to the inclusion of the European species
259 *L. richardsi*. It is important to mention that the biology of *L. elongata* has been poorly studied, and
260 this species has never been reported as carrion-breeder (James & Maslin, 1947; Briggs, 1975; Bolek
261 & Janovy, 2004), possibly behaving only as an obligate parasite of anurans in North America. Thus,
262 *L. elongata* and *L. bufonivora* may be the only two species that exhibit this obligate parasitism
263 behaviour among the genus *Lucilia*. Interestingly, they are placed together as monophyletic sister
264 taxa in both the *cox1* and combined-gene trees.

265 *Lucilia bufonivora* is considered a rare species in England and there are few reports of
266 confirmed toad myiasis cases where it is involved (McDonagh & Stevens, 2011) and adult flies of
267 this species are rarely caught using carrion-baited traps (Arias-Robledo, unpublished data). This
268 may illustrate the highly specific nature of the cues emanating from a living amphibian host that are
269 required to attract *L. bufonivora*, or simply may reflect its restricted distribution and low abundance
270 in the field. In this study, the molecular identification of larval samples extracted from toad myiasis
271 cases as *L. bufonivora* reaffirmed the presence of this obligate parasite in Britain (Fig. 3). A study
272 in Germany suggests that this species is highly variable in its local abundance (Weddeling &
273 Kordges, 2008).

274 Based on mitochondrial sequence data, European specimens of *L. silvarum* were more
275 closely related to *L. richardsi* than to *L. bufonivora*. However, the *efla*-based phylogeny placed
276 *L. richardsi* as a sister species of *L. sericata* outside of the amphibian parasite group of flies, as
277 observed in previous phylogenetic analyses (McDonagh & Stevens, 2011). Although *L. sericata*
278 and *L. silvarum* have been reported as facultative parasites of sheep and amphibians, respectively
279 (McLeod, 1937; Hall, 1948), there are no records of *L. richardsi* being involved in either sheep or
280 toad myiasis. However, Nuorteva (1959) reported that three males of *L. richardsi* were reared from
281 a single case of wound myiasis in a bird (a nightjar). The high similarity of *L. richardsi* with
282 *L. sericata* based on nuclear DNA and with *L. silvarum* based on mitochondrial DNA, might be
283 attributed to introgressive hybridization, however, more detailed studies are required to confirm
284 this. The occurrence of hybridisation has important implications for speciation, and this
285 phenomenon has been reported several times occurring within the genus *Lucilia*, as it is the case of
286 the hybridization between the closely related species *L. sericata* and *L. cuprina* (Stevens & Wall,
287 1996b; Williams & Villet, 2013). Similarly, *Lucilia illustris* and *Lucilia caesar* present very low
288 genetic distances, and they could not be reliably identified using mitochondrial markers, which
289 might result from hybridisation or incomplete lineage sorting (Sonet *et al.*, 2012).

290 In conclusion, it has been suggested that the myiasis habit may have arisen in multiple
291 independent evolutionary events within the subfamily Luciliinae (Stevens, 2003). The results
292 presented here support this and suggest that the *obligate* parasitic habit in the genus *Lucilia* possibly
293 diverged from *L. silvarum*. Further studies that include more specimens of *L. elongata* from
294 different geographical regions are required to explore its molecular identity and to resolve its
295 evolutionary relationships within the broader amphibian parasite group of blowfly species.

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311

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444 **Figure Legends**

445

446 Figure 1. Common toad (*Bufo bufo*) with nasal myiasis due to *Lucilia bufonivora*, Bridgnorth,
447 Shropshire, UK; posterior ends of live 3rd instar larvae are visible within the enlarged wounds at
448 the site of each nostril (photograph courtesy of Dr A. Breed, Animal and Plant Health Agency,
449 Defra, UK).

450

451 Figure 2. Bayesian inference trees constructed from **(a)** the EF1-EF4 region of the nuclear gene
452 *eflα* and **(b)** the mitochondrial gene *coxI*. Posterior probability values are labelled on each node.
453 AUS = Australia, CAN = Canada, CHE = Switzerland, DEU = Germany, GBR or UK = United
454 Kingdom, NLD = The Netherlands, NZL = New Zealand, Suff = Suffolk (UK), USA = United
455 States, WN = Winssen (The Netherlands), Olst = Olst (The Netherlands). * = sequence data from
456 EMBL/GenBank. Lbufo = *L. bufonivora*, Lsilv = *L. silvarum*, Lrich = *L. richardsi*, Lillus =
457 *L. illustris*, Lcae = *L. caesar*, Lamp = *L. ampullacea*, Lmex = *L. mexicana*, Cvic = *Calliphora*
458 *vicina*, Lbufo17 = *L. bufonivora* (Shrewsbury-1).

459

460 Figure 3. Bayesian inference tree constructed from a partitioned dataset of the combined genes *eflα*
461 and *coxI*. Posterior probability values are labelled on each node. AUS = Australia, CAN = Canada,
462 CHE = Switzerland, DEU = Germany, GBR or UK = United Kingdom, NLD = The Netherlands,
463 NZL = New Zealand, Suff = Suffolk (UK), USA = United States, WN = Winssen (The
464 Netherlands), Olst = Olst (The Netherlands). * = sequence data from EMBL/GenBank. Lbufo =
465 *L. bufonivora*, Lsilv = *L. silvarum*, Lrich = *L. richardsi*, Lillus = *L. illustris*, Lcae = *L. caesar*, Lamp
466 = *L. ampullacea*, Lmex = *L. mexicana*, Cvic = *Calliphora vicina*, Lbufo17 = *L. bufonivora*
467 (Shrewsbury-1).

468

469 Table 1. Larval *Lucilia* specimens studied, including the location of collection, name of sample
 470 used for phylogenetic analysis and accession numbers for EMBL/GenBank DNA sequences for
 471 both *cox1* and *ef1α*.

Infestation ID	Larvae analysed	Country/Region of origin	Code	<i>cox1</i>	<i>ef1α</i>
BB016-2	1	Haaksbergen, The Netherlands	L. bufo (NLD1)	FR719161	FR719238
BB016-3	1	Haaksbergen, The Netherlands	L. bufo (NLD2)	FR719161	FR719238
BB016-1	1	Zelhem, The Netherlands	L. bufo (NLD3)	FR719161	FR719238
BB016-4	1	Haaksbergen, The Netherlands	L. bufo (NLD4)	FR719161	FR719238
BBSP1	1	Haaksbergen, The Netherlands	L. bufo (NLD5)	FR719161	FR719238
Friesl-1	1	Friesland, The Netherlands	L. bufo (NLD6)	FR719161	FR719238
Rott-1	1	Rotterdam, The Netherlands	L. bufo(NLD7)	FR719161	FR719238
Oss-Ch-1	1	Ossingen, Switzerland	L. bufo (CHE)	FR719161	FR719238
WV15 6QR-1	1	Bridgnorth, Shropshire, UK	L. bufo (GBR1)	FR719161	FR719238
WV15 6QR-2	1	Bridgnorth, Shropshire, UK	L. bufo (GBR2)	FR719161	FR719238
XT767-16	1	Loughborough, UK	L. bufo (GBR3)	FR719161	FR719238
XT931-16	1	Bridgnorth, Shropshire, UK	L. bufo (GBR4)	FR719161	FR719238
Holk-1	2	Holkam, UK	L. bufo (GBR5 + 6)	FR719161	FR719238
Shrew-446	2	Shrewsbury, UK	L. bufo 17	FR719161	+LT900481
			L. bufo (GBR8)	FR719161	FR719238
Nott-1	2	Nottingham, UK	L. bufo (GBR9 + 10)	FR719161	FR719238
Suff-1	2	Suffolk, UK	L. bufo (Suff1 + 2)*	FR719161	FR719238

472
 473 + = new sequence; * see McDonagh & Stevens (2011)

474
 475

476 Table 2. Larval *Lucilia* specimens studied, including the location of collection, name of sample
 477 used for phylogenetic reconstruction, and accession numbers for GenBank DNA sequences for both
 478 *cox1* and *ef1α*.

479

Species	ID	Country/Region of origin	Code	<i>cox1</i>	<i>ef1α</i>
<i>L. bufonivora</i>	DM	Baden-Württemberg, Germany	L. bufo (DEU1)	FR719161	FR719238
<i>L. bufonivora</i>	DM	Baden-Württemberg, Germany	L. bufo (DEU2)	FR719161	FR719238
<i>L. bufonivora</i>	GAR	Olst, The Netherlands	L. bufo (Olst)	FR719161	FR719238
<i>L. bufonivora</i>	GAR	Winssen, The Netherlands	L. bufo (WN)	FR719161	FR719238
<i>L. elongata</i>	AT	Canada	L. elongata(CAN)	KM858341*	+LT965032
<i>L. silvarum</i>	GAR	Bristol, UK	L. silv (GBR1)	KJ394947	FR719260
<i>L. silvarum</i>	GAR	Bristol, UK	L. silv (GBR2)	KJ394947	FR719260
<i>L. silvarum</i>	GAR	Bristol, UK	L. silv (GBR4)	KJ394947	FR719260
<i>L. silvarum</i>	RLW	San Francisco, USA	L. silv (USA)	FR719259*	FR719259*
<i>L. silvarum</i>	RLW	Sacramento, USA	Lsilv SacrUSA-2	+LT963484	+LT965034
<i>L. silvarum</i>	GAR	Olst, The Netherlands	Lsilv (NLD-1)	+LT963483	FR719253
<i>L. richardsi</i>	GAR	Bristol, UK	L. rich (1)	FR872384	FR719253
<i>L. richardsi</i>	GAR	Bristol, UK	L. rich (2)	KJ394940	FR719253
<i>L. sericata</i>	GAR	Bristol, UK	L. sericata (UK)	AJ417714	+LT965035
<i>L. sericata</i>	JRS	Los Angeles, USA	L. sericata(USA)	AJ417715*	FR719257*
<i>L. cuprina</i>	RLW	Perth, Australia	L. cuprina(AUS)	AJ417707*	FR719245*
<i>L. cuprina</i>	AH/ DMB	Dorie, South Island, New Zealand	L. cuprina NZ)	AJ417706*	FR719244*
<i>L. caesar</i>	GAR	Bristol, UK	L. cae (Bristol-1)	+LT900367	+LT900482
<i>L. illustris</i>	RLW	Somerset, UK	L. illus	FR872384*	FR719253*
<i>L. ampullacea</i>	GAR	Bristol, UK	L. amp (Bristol-2)	+LT963485	+LT965033
<i>L. ampullacea</i>	RLW	Somerset, UK	L. amp	FR719236*	EU925394*
<i>L. mexicana</i>	FAV	Chapingo, Mexico	L. mex (MEX1)	+LT900368	+LT900483
<i>L. mexicana</i>	FAV	Chapingo, Mexico	L. mex (MEX2)	+LT900368	+LT900483
<i>C. vicina</i> [^]	GAR	Switzerland (laboratory reared)	C. vic (CHE)	KJ635728 [#]	FR719219
<i>C. vicina</i>	GAR	Bristol, UK	C. vic (1)	KJ635728	FR719219
<i>C. vicina</i>	GAR	Bristol, UK	C. vic (2)	KJ635728	FR719219

480

481 Adult specimen identification: GAR = Gerardo Arias-Robledo (Bristol, UK), JRS = Jamie Stevens
 482 (Exeter, UK), RLW = Richard Wall (Bristol, UK), FAV = Francisco Arias-Velazquez (Chapingo,
 483 Mexico), DM = Dietrich Mebs (Frankfurt, Germany), AH = Allen Heath (AgResearch, New
 484 Zealand), DMB = Dallas Bishop (AgResearch, New Zealand); AT = Angela Telfer (Guelph,
 485 Canada).

486 + = new sequence; * = sequence data from EMBL/GenBank; ^ = unidentified specimens provided
 487 by G. Guex (Zurich) and identified at University of Exeter by GAR; [#] identity based on 540 bp of
 488 sequence data.

489 Table 3. Amplification and internal sequencing primers used to amplify the two genes studied,
490 including the source of published primers.

491

Gene	Primer	Sequence	Source
<i>ef1α</i>	EF1	ACAGCGACGGTTTGTCTCATGTC	McDonagh et al. (2009)
	EF4	CCTGGTTCAAGGGATGGAA	McDonagh et al. (2009)
<i>cox1</i>	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)

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